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Mitochondrial DNA of Phytophagous Insects as a Molecular Tool for Phylogeographic Study of Host Plants

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Our purpose is to reveal the detailed postglacial colonizing routes of broad-leaved evergreen forests from the refugia in the Quaternary. In this study, to obtain results, we used intraspecific variations in mitochondrial DNA (mtDNA) of phytophagous animals for the phylogeographic study of their host plants. The mtDNA of the phytophagous insect *Curculio hilgendorfi* had a greater amount of intraspecific variation than that of the cpDNA of the plants growing in the same *Castanopsis*-dominant forests. Minimum spanning network of the mtDNA haplotypes showed that haplotypes of primordial origin were geographically quite widespread; whereas those of recent origin were restricted to one or a few localities. Therefore, the phylogenetic information among the mtDNA haplotypes will be useful for the phylogeographic study of *Cu. hilgendorfi*. Such patterns were not seen in plant cpDNA haplotypes.

The host-parasite relationship between *Castanopsis* and *Curculio hilgendorfi* also proved to be very tight in this study. If the phylogeographic congruency between phytophagous insects and their host plants could be demonstrated, information about intraspecific mtDNA variation in the phytophagous insect *Cu. hilgendorfi* would be very useful for the phylogeographic study of *Castanopsis* type broad-leaved evergreen forests in Japan.

Key words: broad-leaved evergreen forest, *Curculio*, glacial refugia, mitochondrial DNA, phylogeography, phytophagous insects, seed-parasitic insects

With the development of molecular methods, it has become possible to investigate the geographical distribution of genetic variation and to deduce intraspecific phylogeographic structures (Avise *et al.* 1987, Avise 2000). Intraspecific variation of chloroplast DNA (cpDNA) has often been used in the phylogeographic analysis of plants (Tsumura *et al.* 1994, Demesure *et al.* 1996, Dumolin-Lapègue *et al.* 1997, Soltis *et al.* 1997, Fujii *et al.* 1997, 2002, Ferris *et al.* 1998, Abbott *et al.* 2000, Petit *et al.* 2003, Aoki *et al.* 2003, 2004a, b, Griffin *et al.* 2004). Plant cpDNA is effectively haploid, and is present in multiple copies and does not recombine. Another superior feature of cpDNA is its small effective population size, which is one-quarter that of nuclear DNA (Moore 1995).

Since genetic drift excludes genetic variation from a gene pool more effectively in cpDNA than in nuclear DNA, it is less feasible that ancestral polymorphism and linage sorting in cpDNA would cause disagreement between a gene tree and a species tree (Moore 1995). Moreover, cpDNA is usually maternally inherited in angiosperm species and hence transmitted only by seeds. The restricted

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TABLE 1. Number of polymorphic sites, haplotype diversity and nucleotide diversity in the 3 coding regions of *Curculio* mtDNA

	Number of samples	Number of polymorphic sites	Number of found haplotypes	Haplotype diversity	Nucleotide diversity	Theta per site
Clade I (*Curculio hilgendorfi*)*	71	120	44	0.957	0.00472	0.00870
Clade I-2, Clade I-3	69	74	42	0.954	0.00384	0.00540
Clade II (*Curculio sikkimensis*)	18	62	18	1.000	0.00462	0.00631
Clade I, Clade II	89	489	62	0.973	0.04965	0.03387
Total	90	607	63	0.973	0.05140	0.04195

*See Fig. 3.

seed dispersal in plants than pollen dispersal or migration in animals facilitates the conservation of geographic patterns of cytoplasmic genetic variation that developed as a result of historical migration (Cruzan & Templeton 2000). Therefore, cpDNA is well suited for the reconstruction of past migrational routes of plant species (Petit *et al.* 1993). However, the molecular evolutionary rate of cpDNA has been reported to be relatively slow ($2x10^{-11}$ to $5x10^{-10}$ substitutions per site per year) at the nucleotide sequence level (Palmer 1987, Wolfe *et al.* 1987, Bousquet *et al.* 1992, Chase *et al.* 1993, Frascaria *et al.* 1993, Hasebe *et al.* 1998, Yatabe *et al.* 1999). The molecular evolutionary rate of non-coding cpDNA regions (*trnT-trnL, trnL-trnF, atpB-rbcL, rps16, trnG, psbC-trnS, trnG-trnfM, trnW-trnP, petB, petD-rpoA, rpl16*) that are often used for phylogeographic analyses was also slow, i.e., $1.2x10^{-10}$ (including indels) and $7.7x10^{-11}$ (nucleotide substitutions only) substitutions per site per year (Aoki *et al.* unpublished data), or one nucleotide substitution every 2 million years per site on average. As a matter of course, relatively low levels of cpDNA variation are usually found within species. Most of the cpDNA haplotypes found in one plant species probably originated long before the Quaternary period (2 million years ago), and thus the phylogenetic relationships among the cpDNA haplotypes do not reflect any recent (that is, after the last glacial maximum of 20,000 years ago) process of range expansion of plants. In order to investigate recent phylogeographic processes, for example to reveal the detailed postglacial colonizing routes from the refugia in the Quaternary, we need to use DNA markers with a faster evolutionary rate. In this study, we proposed to use intraspecific variation in mitochondrial DNA (mtDNA) of phytophagous animals for the phylogeographic study of their host plants.

It has been estimated that the synonymous rate of sequence change is considerably faster in animal mtDNA than in plant cpDNA, mtDNA, or even nuclear DNA (Brown *et al.* 1982, Palmer 1987, Wolfe *et al.* 1987, Mitton 1994). The rates of synonymous substitution were estimated to be 3.6-$9.4x10^{-8}$ per site per year for mammalian species (Brown *et al.* 1982) and 1-$3x10^{-8}$ per site per year for *Drosophila* (Satta *et al.* 1987). The substitution rate of animal mtDNA appears to be about 100 times the rate in plant cpDNA or mtDNA. If information of intraspecific mtDNA variation in animals living specifically in a few numbers of plant species growing in a particular type of forest can be available for the phylogeographic study of those plants, it would allow us to avoid the limitation of low levels of within-species variation in plant cpDNA. The use of DNA markers with a faster evolutionary rate would enable us to detect more intraspecific variations that originated more recently.

In plant cpDNA, most of the cpDNA haplotypes originated long ago, and thus ancient haplotypes and relatively recent haplotypes are expected to exist together in a population. This was in fact the case in our previous study examining intraspecific cpDNA variation of 6 plant species growing in Japanese broad-leaved evergreen forests (Aoki *et al.* 2004a). Using animal mtDNA as the genetic mark-

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ers for phylogeographic studies of host plants, we may be able to examine even recent postglacial colonizing processes of plants by using the phylogenetic relationship among haplotypes more effectively when the animals and the plants always moved together.

Nested clade analyses (Templeton *et al.* 1987, 1995) use both information on geographic distribution patterns of the haplotypes and the phylogenetic relationships among the haplotypes to test hypotheses concerning historical processes that have led to the present geographical distributions. The largest merit of this method lies in its objective phylogeographic inference concerning specific historical processes, for example, past population fragmentation, range expansion, or colonization through statistical tests. Under the restricted gene flow model, an association between a haplotype's age and its distribution ranges has been predicted from coalescence theory and the results of computer simulations of dispersal/mutation processes (Hudson 1990, Neigel *et al.* 1991). These analyses predict that most anciently originated haplotypes should be located at the central position of a network and be geographically widespread, whereas most recently originated haplotypes should be at the tips of the gene tree and be geographically localized in narrower regions (Golding 1987, Castelloe & Templeton 1994, Templeton *et al.* 1995). Such predictions can be realized only when we use DNA markers indicating that a species expanded its distribution range while producing mutations on the markers. Actually, nested clade analysis of insect (i.e., alpine butterfly) mtDNA suggested that the species experienced repeated cycles of population expansion and fragmentation, a finding which corresponded with paleoclimatic data on habitat expansion and contraction over the past 400,000 years (DeChaine & Martin 2004). If we can use intraspecific mtDNA variation in phytophagous animals for the phylogeographic study of their host plants, we may be able to specify recent historical processes that produced the present geographical distribution of plants, even after the last glacial maximum, if they have tight association.

Taberlet *et al.* (1998) and Hewitt (1999) analyzed 10 taxa of both animals and plants for their phylogeographic study. However, the animal and plant species chosen in these studies live independently, so the intention was not to perform phylogeographic studies of plants using molecular information of animals. In the present study, we chose seed-parasitic weevil species inhabiting specifically *Castanopsis*-dominant forests in a broad-leaved evergreen zone because we have worked on the phylogeography of 6 plant species growing only in this type of forest using cpDNA variation as genetic markers (Aoki *et al.* 2004a).

The weevil *Curculio hilgendorfi* Harold (Coleoptera: Curculionidae) lays eggs and feeds specifically on the acorns of *Castanopsis* (Fagaceae) in Japan (Hayashi *et al.* 1984). The host plant genus *Castanopsis* in Japan consists of two species and one variety: *Ca. cuspidata, Ca. sieboldii* and *Ca. sieboldii* var. *lutchuensis* (Yamazaki & Mashiba 1987a, b). *Ca. sieboldii* var. *lutchuensis* is distributed in Ryukyu Islands. Four species closely related to *Curculio hilgendorfi, Cu. sikkimensis, Cu. dentipes, Cu. robustus* and *Cu. conjugalis*, deposit their eggs on or in the acorns of several Fagaceae species (*Quercus, Lithocarpus*, and *Castanea*). During autumn, female weevils drill acorns with their long proboscis and lay an egg in the acorn through the hole with their long ovipositor. Larvae feed in the acorn, bore an exit hole, and leave the acorn to burrow into the ground and overwinter (Morimoto 1986). We expect that we can obtain enough *Curculio* larvae in many localities to suffice for our study by simply collecting the acorns of host species.

In this study, we addressed three specific questions for assessing whether or not the intraspecific mtDNA variation in the phytophagous insect *Curculio hilgendorfi* would be more useful than that of cpDNA in plant species. We did this in order to

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study recent phylogeographic processes of *Castanopsis* type broad-leaved evergreen forests in Japan. These questions were: (1) Is the host-parasite relationship between *Cu. hilgendorfi* and its host plant species as tight as it has been reported to be? (2) Can a considerably larger amount of intraspecific variations than those found in the cpDNAs of the 6 plant species growing in *Castan-opsis*-dominant forests examined in our previous study actually be detected in the mtDNA of *Cu. hilgendorfi*? and (3) Is the observed mtDNA haplotypes of this weevil more geographically structured than the cpDNA haplotypes in the 6 plant species?

Materials and Methods

Insect materials

Acorns of *Castanopsis sieboldii, Ca. sieboldii* var. *lutchuensis* and *Ca. cuspidata* were collected from 45 localities in Japan during autumn 2002 and 2003, covering most of the geographical distribution range of these species in Japan. We tried to collect acorns from several trees in each locality. A small number of acorns of *Quercus* and *Lithocarpus* were also collected for comparison. The collection sites for each plant species are listed in Appendix 1. The obtained acorns were kept separately in plastic bottles. The larvae of *Curculio* weevils were collected every 3 to 5 days until December. Most of the *Curculio* weevil larvae were preserved in 99% ethanol for DNA analysis. After almost all the larvae had left the acorns, we counted the number of exit holes in the acorns from each tree. The number of exit holes in the acorns can be more precisely estimated as rate of infestation than the number of larvae from the acorns because we also collected acorns which the larvae had already left through exit holes long before our collection.

Matured insects of the genus *Curculio* have a similar external morphology; the key diagnostic characteristics for their species classification are those of the genitalia, especially the shape of the copulatory piece. For species identification, the rest of the obtained larvae were kept with wet vermiculite in the dark from autumn 2002 to summer 2003. The legs of emerged males were preserved in 99% ethanol for DNA analysis, and the remaining bodies were dissected for species identification. The identification method using shapes of copulatory pieces follows Hayashi *et al.* (1984).

DNA sequencing

Total DNA was extracted from thoracic muscles of larvae (or leg muscles of adults) using standard Proteinase K/SDS digestion and phenol-chloroform extraction. DNA fragments of the coding regions of mitochondrial cytochrome oxidase subunit I (COI), II (COII), and NADH dehydrogenase (ND5) were amplified using the following primers: 5'-GGATCACCTGATATAGCATTCCC-3' and 5'-CCTAAAAAATGTTGTGGGAAAAAGG-3' for COI, 5'-CAATGATTCCCATTATTCACTGG-3' and 5'-CCACAAATTTCTGAACATTGACC-3' for COII according to Satta & Takahata (1990), and 5'-CCTGTTTCTGCTTTAGTTCA-3' and 5'-GTCATACTCTAAATATAAGCTA-3' for ND5 according to Su *et al.* (1996), respectively. The polymerase chain reaction (PCR) products were purified using a QIAquick Gel Extraction Kit (Qiagen) after electrophoresis in 1.0% agarose gels, and they were then used as templates for direct sequencing.

The sequencing reactions were prepared using a Big Dye terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Foster City, Calif., USA). The reaction mixtures were analyzed on an Applied Biosystems model 3100 automated sequencer (Perkin Elmer Applied Biosystems). The sequences were aligned using Sequence Navigator software (Perkin Elmer Applied Biosystems) and SeqPup version 0.6 (Gilbert 1996). Calculations of haplotype diversity, nucleotide diversity (Tajima 1983), and theta per site (Watterson 1975) were carried out using DnaSP version 3.51 (Rozas & Rozas 2000).

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Phylogenetic analysis

Phylogenetic analysis was performed by the Neighbor-Joining (NJ) method (Saitou & Nei 1987) using CLUSTAL X (Thompson *et al.* 1997). In calculating the distance matrix, we implemented Kimura's 2-parameter model of nucleotide substitutions (Kimura 1980) using the same computer program. A bootstrap analysis with 1,000 replications was performed to estimate the reliability for various clades.

In addition to reconstruction of phylogenetic tree, a minimum spanning network was constructed using the statistical parsimony approach described by Templeton *et al.* (1992) as implemented in TCS version 1.13 (Clement *et al.* 2000).

Results

The rate of infestation by Curculio *in the acorns of Fagaceae*

For the sample collection in 2002, we obtained 1,372 individual insect larvae from a total of 24,633 acorns of *Castanopsis* and 862 acorns of *Quercus* and *Lithocarpus*, of which 1,331 individuals were identified to be *Curculio* larvae from their larval morphology. In most of the collection sites, some bored acorns were found from the collection day to 1 month after collection. The ratio of these bored acorns per collected acorns in each locality was 1 to 37 percent. Each acorn of *Castanopsis* had only one exit hole, and thus, there was only one larva growing in an acorn. To estimate the rate of infestation in 2002, we calculated the ratio of bored acorns per collected acorns under each tree of *Castanopsis sieboldii* (including *Ca. sieboldii* var. *lutchuensis*) and *Ca. cuspidata* (Fig. 1). The results showed that the rates of infestation were 5 to 20 percent in most localities. Highest infestation rates (35, 43, and 47 percent) were observed in acorns from the trees in locality No. 22.

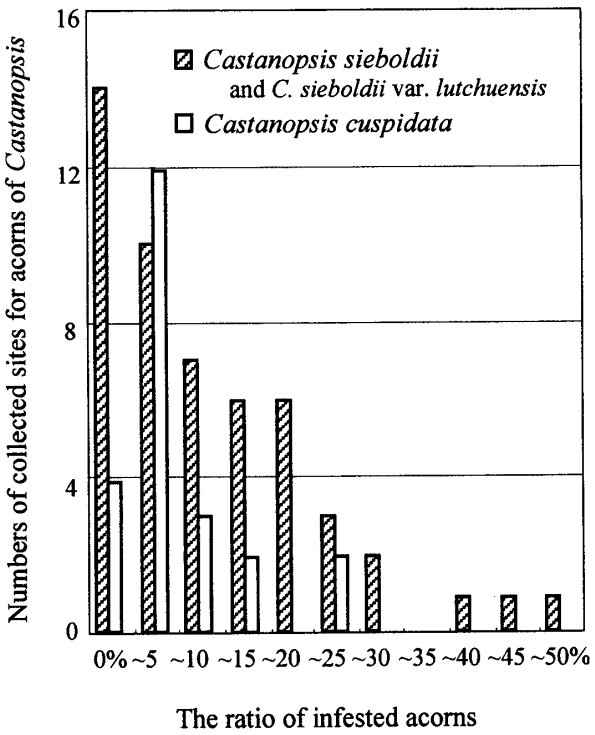

FIG. 1. The ratio of infested acorns to collected acorns under each *Castanopsis sieboldii*, *Ca. sieboldii* var. *lutchuensis* and *Ca. cuspidata* tree.

Species identification of the emerged males based on the shape of their copulatory pieces

We kept 350 larvae from acorns of *Castanopsis* and 70 from those of *Quercus* and *Lithocarpus* during autumn 2002 to summer 2003. Most of the larvae growing in vermiculite died of fungi attack before pupating or were still larval state in summer 2003. Weevil larvae were reported to show a high susceptibility to infection by entomogenous fungi (Saito & Suzuki 1982). We obtained 13 pupae from acorns of *Castanopsis* and 11 from those of *Quercus* and *Lithocarpus* in July in 2003, of which some pupae died before emerging. In this study, 2 adult males and 3 females emerged from the larvae collected from acorns of *Castanopsis sieboldii* and 5 adult males and 3 females from those of *Quercus crispula* and *Q. gilva*. The shapes of copulatory pieces observed in the adult males are shown in Fig. 2. Two individuals from the acorns of *C. sieboldii* were identified as *Curculio hilgendorfi*, and 3 individuals from *Q. crispula* and *Q. gilva*

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were identified as *Cu. sikkimensis* based on the shapes of their copulatory pieces.

Nucleotide sequence variation in Curculio *mtDNA*

The nucleotide sequences of the 3 coding regions (2,856 bp) of 81 *Curculio* larvae collected from acorns of *Castanopsis* and 10 larvae from acorns of the other 6 host plant species were determined. The obtained sequences have been deposited in the DNA Databank of Japan (DDBJ) under the accession numbers AB177407-AB177530. Sequence variability observed in the *Curculio* mtDNA is summarized in Table 1. The calculated values of haplotype diversity, nucleotide diversity, and theta per site are given in Table 1. Six hundred and seven variable sites were found in the mtDNA regions of the 90 individuals of *Curculio*, and 63 haplotypes in total were detected.

Phylogenetic relationships among the Curculio *mtDNA haplotypes*

Based on the 2,856 nucleotide sequences of mtDNA, we obtained the NJ tree among the 63 haplotypes found in the larvae of *Curculio*. The NJ tree with bootstrap values is shown in Fig. 3. Haplotype labels alphabetically denote their relative frequencies in each insect species from capital letters, small letters, to Greek letters, with the A-haplotype being the commonest haplotype, and then B, C, ... Y, Z, a, b, c, ... y, z, α, β, γ, ..., and so on.

The obtained NJ tree consists of two highly supported clades (clade I and clade II). Clade I is comprised of most of the haplotypes found in the *Curculio* larvae from the acorns of *Castanopsis sieboldii*, *Ca. sieboldii* var. *lutchuensis*, and *Ca. cuspidata*, whereas clade II is comprised of the haplo-

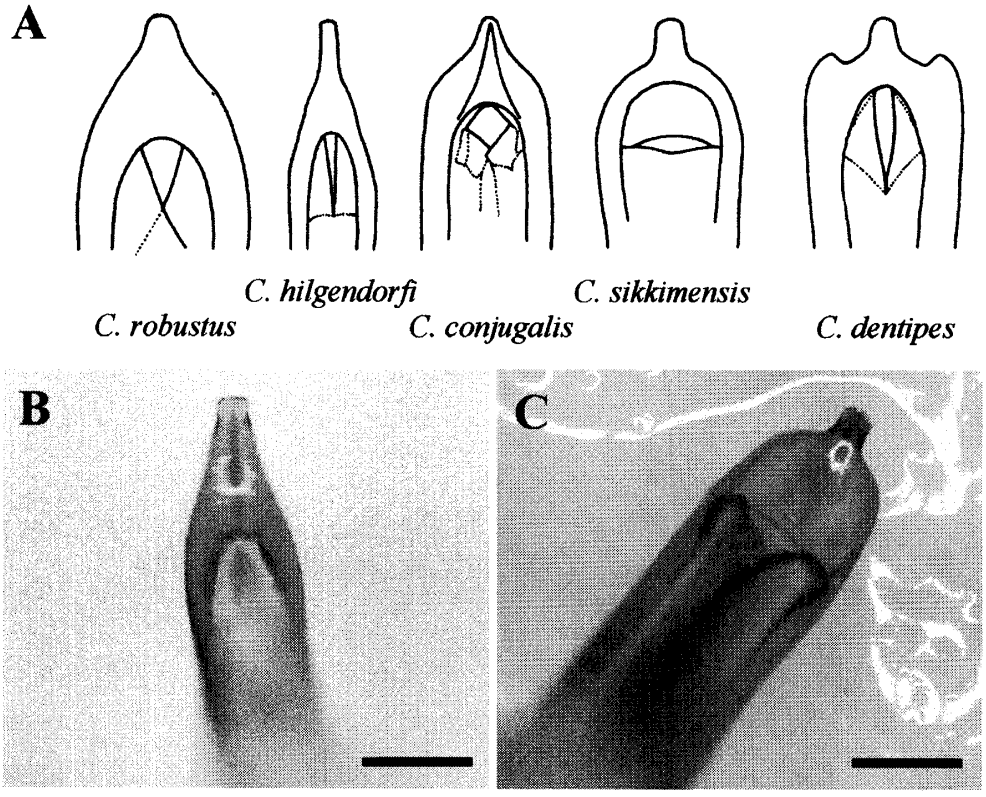

FIG. 2. A-C The shapes of copulatory pieces of *Curculio*. A. Copulatory pieces of five species closely related to *Curculio hilgendorfi* (Hayashi *et al.* 1984). B. The observed copulatory piece of the emerged male grown from the larvae that appeared from 2 acorns of *Castanopsis sieboldii* in Fukuoka (Locality No. 22). It was identified as *Cu. hilgendorfi* based on the shape. C. The observed copulatory piece of the emerged male grown from the larvae that appeared from 2 acorns of *Quercus crispula* in Shiga (Locality No. 9) and 1 acorn of *Q. gilva* in Miyazaki (Locality No. 29). The males were identified as *Cu. sikkimensis* based on the shape. Bar, 3mm.

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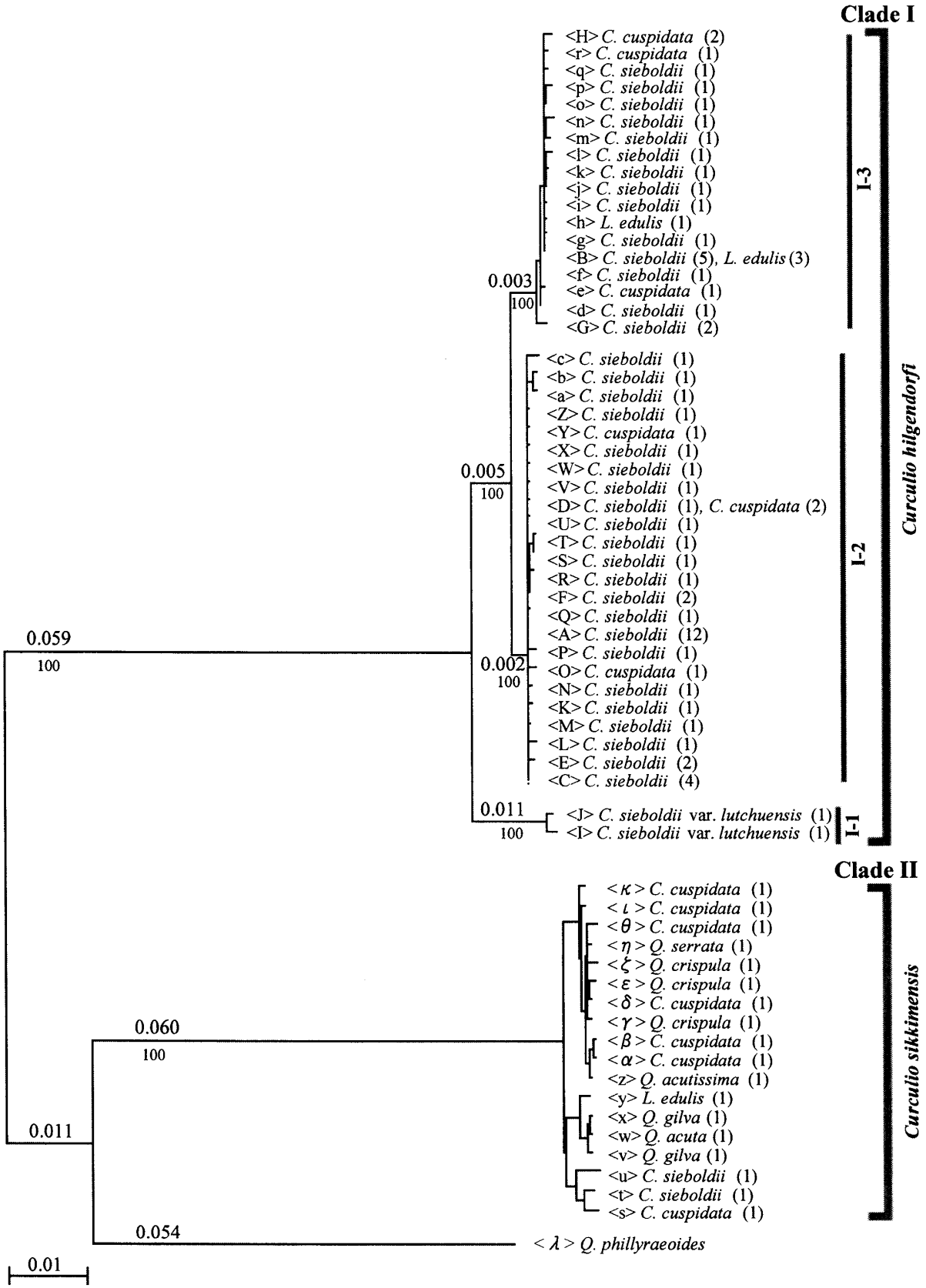

FIG. 3. The Neighbor-Joining tree among the mtDNA haplotypes found from *Curculio* based on their nucleotide sequence data. The numbers above and under branches are evolutionary distances and bootstrap percentages, respectively. Scale bar indicates substitutions per site. Species names represent host plant species of *Curculio*. Numbers in parentheses represent the number of individuals belonging to each haplotype.

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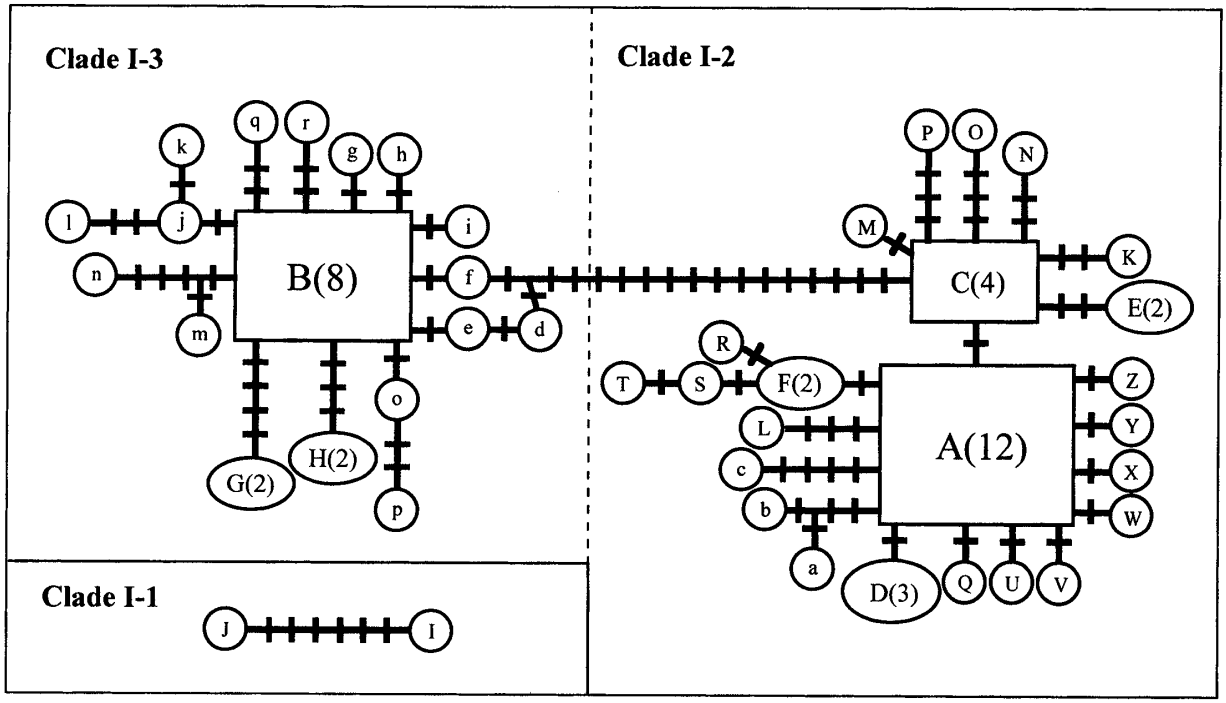

FIG. 4. Minimum spanning network as estimated from statistical parsimony (Templeton *et al.* 1992). Mutations are represented as bars. Haplotypes estimated to be the most likely common ancestor within each of the sub-clades are displayed as squares. Numbers in parentheses represent the number of individuals more than 2 individuals belonging to each haplotype.

types found in those from the acorns of *Quercus serrata*, *Q. crispula*, *Q. acutissima*, *Q. gilva*, *Q. acuta*, and *Q. phillyraeoides*. The haplotypes t, u, α, δ, θ, ι, and κ found from the acorns of *Castanopsis* collected in Kyoto, Nara, and Okayama are also contained in clade II. The haplotypes found in the acorns of *Lithocarpus edulis* (haplotypes B, h, and y) were contained in both clade I and II.

Sequence variability, haplotype diversity, nucleotide diversity, and theta per site among the individuals in each clade are given in Table 1. One hundred and twenty variable sites were found among the 44 haplotypes (71 individuals) comprising clade I. Among the 18 haplotypes (18 individuals) comprising clade II, 62 variable sites were found. Clade I is comprised of three sub-clades (Fig. 3; clade I-1, clade I-2, and clade I-3). Clade I-1 which were consisted of the haplotypes found only from Ryukyu is located at the basal most position of clade I.

The existence of the same three sub-clades is indicated also from the minimum spanning network (Fig. 4). Sub-clade I-2 seems to comprise two subgroups, and displays defined central haplotype, A and C. These haplotypes were found in 12 and 4 individuals, respectively, while the remaining haplotypes were found in several individuals only. Sub-clade I-3 displays a clearly defined central haplotype, B. This haplotype was found in 8 individuals, while the remaining haplotypes were found in one or two individuals only.

Geographic distribution of the haplotypes in Curculio hilgendorfi

The geographic distribution of the mtDNA haplotypes in clade I (including 2 adult individuals identified as *Curculio hilgendorfi*) is shown in Fig. 5. Sub-clade I-1 consists of the haplotypes found on Ishigaki Island in Ryukyu, sub-clade I-2 consists of the haplotypes found in Kyushu and the western part of the Chugoku and Shikoku districts, and sub-clade I-3 consists of the haplotypes distributed in the eastern part of the Chugoku, Shikoku, Kinki, and Kantou districts. More than one haplotype was

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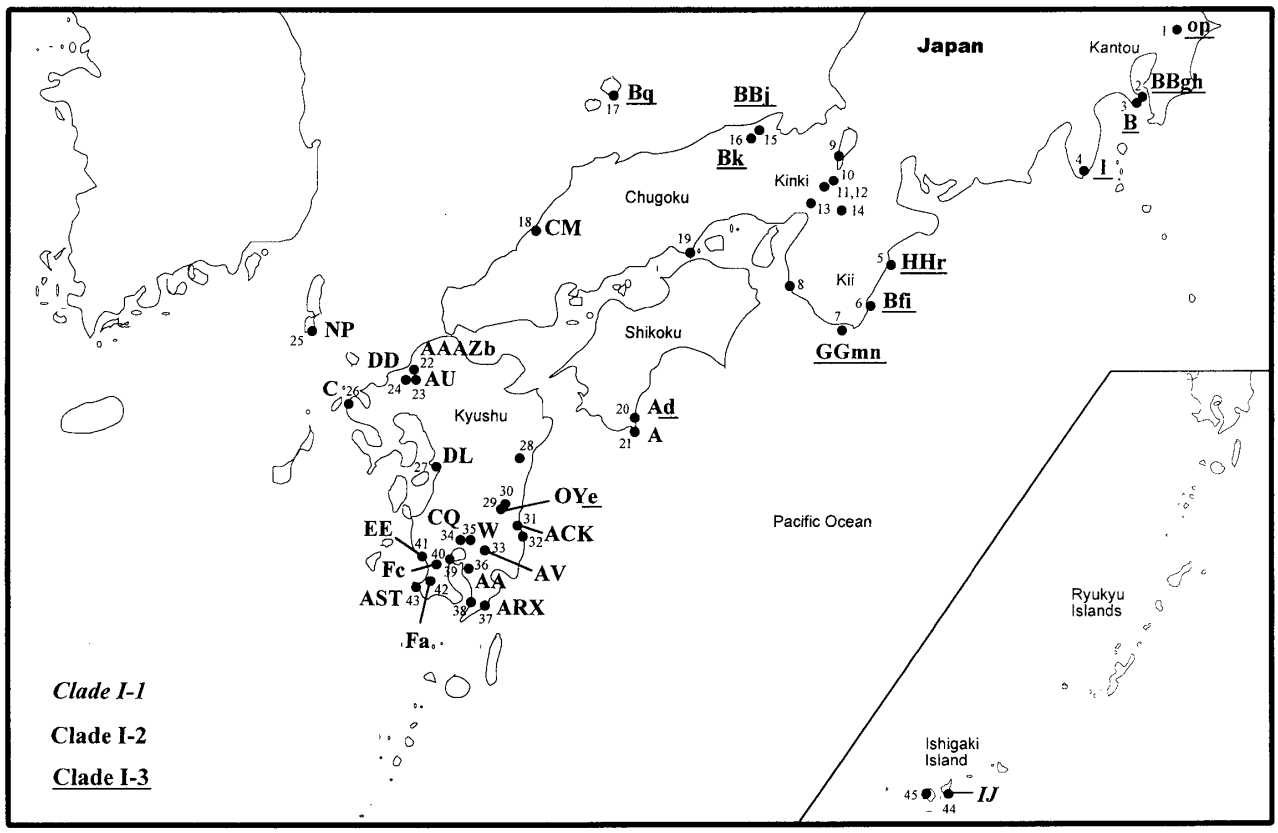

FIG. 5. Geographic distribution of the mtDNA haplotypes found of *Curculio hilgendorfi* in clade I of Fig. 3. Numbers in figure correspond to the localities in Appendix 1. Letters represent the mtDNA haplotype of the individuals.

found in most localities except locality No. 24, 36, and 41 where only one haplotype was found.

Haplotypes located at the central position of the minimum spanning network, for example haplotype A and C in sub-clade I-2 and haplotype B in sub-clade I-3, were geographically widespread. In contrast, most of the haplotypes located at the tips of the network were found only in one individual and were geographically restricted to one locality.

Discussion

The host-parasite relationship between Curculio *and its host plants*

We examined mtDNA variation of 90 *Curculio* larvae obtained from the acorns of 9 plant species (*i.e.*, *Castanopsis, Quercus* and *Lithocarpus*). Most of the *Curculio* belonging to the clade (clade I in Fig. 3) with the adult individuals of *Cu. hilgendorfi* were obtained from the acorns of *Ca. sieboldii* (in-cluding *Ca. sieboldii* var. *lutchuensis*), and *Ca. cuspidata*, both of which are very closely related to each other and sometimes difficult to tell apart (Yamanaka 1966, Yamada & Miyaura 2003). The larvae of *Cu. hilgendorfi* were also obtained from *Lithocarpus*, but the number from this plant source was very small. *Lithocarpus* and *Castanopsis* were growing in the same broad-leaved evergreen zone, but the center of geographic distribution of *Lithocarpus* is in coastal areas. In contrast, the mtDNA clade with the adults of *Cu. sikkimensis* (clade II in Fig. 3) was obtained from acorns of several Fagaceae species which grow in the various type forests of cool - warm temperate zones. These results suggested that parasite insects *Cu. hilgendorfi* and host plants *Castanopsis* have a relatively tight association with each other.

Intraspecific mtDNA variation in Curculio hilgendorfi

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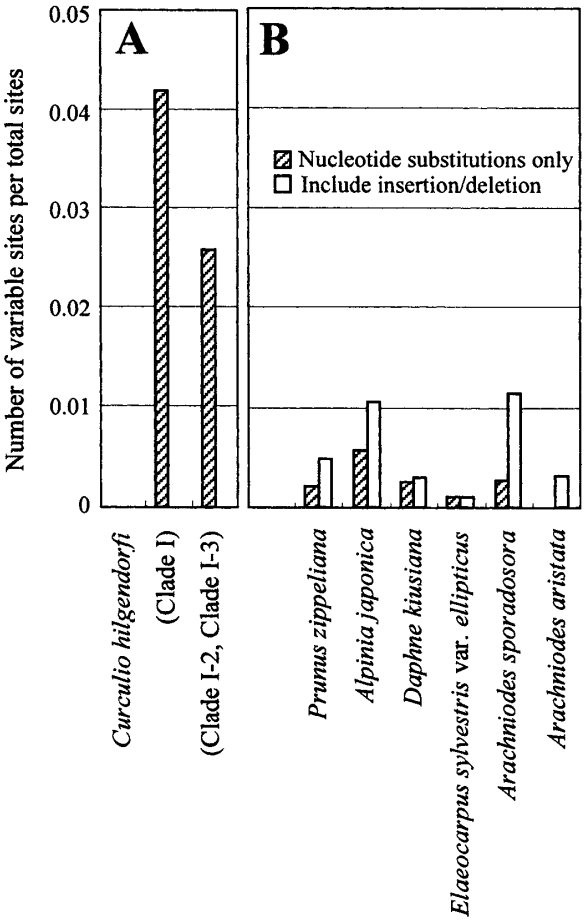

FIG. 6. A. Intraspecific variation in 3 coding regions of mtDNA of *Curculio hilgendorfi*. B. Intraspecific variation in 14 non-coding regions of cpDNA among 6 plant species of the component species of broad-leaved evergreen forests in Japan (Aoki *et al.* 2004a).

Based on the taxonomical identification of the emerged males, we determined that the 2 adult males contained in clade I were *Curculio hilgendorfi* and the 3 adult males contained in clade II were *Cu. sikkimensis*. These two clades were highly supported both by NJ tree and minimum spanning network. In the following discussion, we tentatively regard individuals contained in clade I as *Cu. hilgendorfi* and those in clade II as *Cu. sikkimensis*, even though we know that we must examine more adult males for accurate taxonomical identification.

The results (Figs. 5, 6) showed that much intraspecific variation of mtDNA in *Curculio hilgendorfi* could be found, even after excluding unique haplotypes found from Ryukyu Islands. The amount of intraspecific variation of mtDNA found in *Cu. hilgendorfi* was much higher than that in cpDNA in the 6 plant species reported in Aoki *et al.* (2004a) (Fig. 6). We should take into consideration that the 6 plant species analyzed in Aoki *et al.* (2004a) were selected due to their higher intraspecific variation in cpDNA from 41 component plant species of broad-leaved evergreen forest (Aoki *et al.* 2003), whereas *Cu. hilgendorfi* was not the insect material chosen for the high level of intraspecific variation in its mtDNAs. The results of the present study confirmed that the insect mtDNA has generally much greater intraspecific variation than does the plant cpDNA.

Geographic distribution of haplotypes in Curculio hilgendorfi

The geographic distribution patterns of mtDNA haplotypes in *Curculio hilgendorfi* were clearly structured. The mtDNA haplotypes consisting of sub-clade I-2 and sub-clade I-3 were geographically distributed separately. The geographical boundary between the two groups of haplotypes lies in the Chugoku-Shikoku region (Fig. 5).

Haplotypes located at the central position of the minimum spanning network (i.e., haplotypes A, B, and C) were geographically widespread, whereas most of the haplotypes located at the tips of the network were geographically restricted to one locality. Under the assumption that a species expands its distribution range while producing new mutations in mtDNA, the most ancient haplotypes, which are located at the central position of the minimum spanning network, should be geographically widespread, whereas the most recently originated haplotypes, which are at the tips of the network, should be localized in geographically narrow regions (Golding 1987, Castelloe & Templeton 1994, Templeton *et al.* 1995). Our previous study (Aoki *et al.* 2004a) showed no clear relationships between phylogenetic and geographic distances among the cpDNA

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haplotypes found in the 6 plant species examined. In this study, we observed expected patterns mentioned above in the obtained minimum spanning network and the geographical distribution pattern of the mtDNA haplotypes of *Curculio hilgendorfi*.

Conclusion and Perspective

The mtDNA of *Curculio hilgendorfi* actually proved to have a greater amount of intraspecific variation than that found in the cpDNA of plants distributed in the same geographic region. Furthermore, we observed that anciently originated haplotypes were geographically widespread, whereas recently originated ones were restricted to one or a few localities. Therefore, the phylogenetic information among the mtDNA haplotypes will also be useful for phylogeographic study of *Cu. hilgendorfi*, which could not be used for those of cpDNA in plant species. Analyzing mtDNA variations of *Cu. hilgendorfi* in more samples and from more localities and using nested clade analyses (Templeton *et al.* 1987, 1995) will enable us to more precisely specify recent historical processes affecting the geographic structure of mtDNA haplotypes of this insect species.

We investigated the mtDNA variations and phylogeographic structures in phytophagous insects in order to reconstruct a recent population history of their host plants. The relationship between parasite insects *Curculio hilgendorfi* and host plants *Castanopsis* also proved to be very tight in this study. Therefore, it is likely that phytophagous insects currently living specifically on particular host plants previously migrated with their host plants during climatic change in the Quaternary. Of course, it is also possible that host change might have occurred, host plants and parasitic animals moved differently or that the present gene flow between animal populations is strongly affecting their genetic structure. Our succeeding investigation will be even more successful than the present one if we can show that host plants and parasitic insects have moved together in the past. In this present study, we were unable to directly compare phylogeographic patterns of the phytophagous weevil *Cu. hilgendorfi* to those of host plants *Castanopsis* due to extremely low intraspecific variations in the *Castanopsis* cpDNA. In future studies, we are planning to detect intraspecific variations of host plants by using seven microsatellite markers in *Ca. cuspidata* developed by Ueno *et al.* (2000). We then plan to compare the obtained phylogeographic patterns of the host plants with the mtDNA patterns of phytophagous insects examined in this study. If the phylogeographic congruency between phytophagous insects and their host plants is demonstrated, information about intraspecific mtDNA variations in the phytophagous insect *Cu. hilgendorfi* can be very useful for the phylogeographic study of *Castanopsis* type broad-leaved evergreen forests in Japan.

In addition, mtDNA variations in other host plant-specific weevils or phytophagous insects might also be applicable to the phylogeographic study of host plants growing in a particular type of forest, as long as they have a tight host-parasite association. Even more applications of our results may be possible in the future. We may be able to reveal historical processes that generated present geographic distributions of particular forests from several million years ago up to the recent period (such as after the last glacial maximum). We would do this by comparing the phylogeographic patterns among several phytophagous insects using mtDNA variations and finding the common patterns of geographic distribution of mtDNA haplotypes, as well as those of several plant species using cpDNA variations.

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APPENDIX 1. Collction sites of acorns from Fagaceae plants

No.	Localities	Host species	Collector	Date
1	Narita, Chiba, Japan	*Castanopsis sieboldii*	T. Kiuchi	03.11.5
2	Kannonzaki, Kanagawa, Japan	*Castanopsis sieboldii*	Y. Omae	03.10.01
		Lithocarpus edulis	Y. Omae	03.10.01
3	Takeyama, Kanagawa, Japan	*Lithocarpus edulis*	Y. Omae	03.10.15
4	Minamiizu, Shizuoka, Japan	*Castanopsis sieboldii*	T. Sakura	03.11.3
5	Kuki, Mie, Japan	*Castanopsis cuspidata*	K. Aoki	02.10.24
6	Ukui, Wakayama, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.24
7	Esusaki, Wakayama, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.23
		Quercus phillyraeoides	K. Aoki	02.10.23
8	Minoshima, Wakayama, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.23
		Quercus acutissima	K. Aoki	02.10.23
		Quercus serrata	K. Aoki	02.10.23
9	Mt. Hira, Shiga, Japan	*Quercus crispula*	K. Aoki	02.10.6
10	Kiyomizu, Kyoto, Japan	*Castanopsis cuspidata*	K. Aoki	02.10.26
11	Yoshida, Kyoto, Japan	*Castanopsis cuspidata*	K. Aoki	02.10.22
		Quercus serrata	K. Aoki	02.10.9
12	Oiwake, Kyoto, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.3
		Quercus glauca	K. Aoki	02.10.3
13	Toyono, Osaka, Japan	*Castanopsis cuspidata*	K. Asami	02.11.13
14	Kasuga, Nara, Japan	*Castanopsis cuspidata*	K. Aoki	03.10.26
15	Kehi, Kinosaki, Hyogo, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.30
16	Onsenji, Kinosaki, Hyogo, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.30
		Quercus glauca	K. Aoki	02.10.30
17	Kishihama, Oki Isl., Shimane, Japan	*Castanopsis sieboldii*	K. Aoki	03.11.5
18	Shimokou, Shimane, Japan	*Castanopsis sieboldii*	K. Aoki	03.11.7
19	Kanayamaji, Okayama, Japan	*Castanopsis sieboldii*	S. Kariyama	03.11.1
20	Nuno, Kochi, Japan	*Castanopsis sieboldii*	K. Aoki	03.10.22
21	Mt. Shiraou, Kochi, Japan	*Castanopsis sieboldii*	K. Aoki	03.10.21
22	Kashii, Fukuoka, Japan	*Castanopsis sieboldii*	K. Aoki	02.11.6
		Quercus gilva	K. Aoki	02.11.6
23	Kasuga, Fukuoka, Japan	*Castanopsis sieboldii*	K. Aoki	02.11.6
		Quercus gilva	K. Aoki	02.11.6
24	Minami park, Fukuoka, Japan	*Castanopsis cuspidata*	K. Aoki	02.11.6
25	Mt.Tatera, Tsushima Isl., Nagasaki, Japan	*Castanopsis sieboldii*	S. Morinaga	03.11.5
26	Tabira, Nagasaki, Japan	*Castanopsis sieboldii*	A. Nakano	03.10.27
27	Sumiyoshi, Kumamoto, Japan	*Castanopsis sieboldii*	S. Morinaga	03.10.29
28	Nobeoka, Miyazaki, Japan	*Quercus gilva*	T. Minamitani	02.10.24
29	Kawanaka, Aya, Miyazaki, Japan	*Castanopsis cuspidata*	K. Aoki	02.10.14
		Castanopsis cuspidata	T. Minamitani	02.11.13
		Quercus gilva	T. Minamitani	02.11.13
30	Mt. Omori, Aya, Miyazaki, Japan	*Quercus acuta*	T. Minamitani	02.10.23
		Lithocarpus edulis	T. Minamitani	02.10.23
31	Kamikitakata, Miyazaki, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.15
		Castanopsis cuspidata	K. Aoki	02.10.15
		Quercus glauca	K. Aoki	02.10.15
32	Takasaki, Miyazaki, Japan	*Castanopsis cuspidata*	K. Aoki	02.10.15
33	Tsumagirishima, Miyazaki, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.14
		Castanopsis cuspidata	K. Aoki	02.10.14
34	Kirishima, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.14
		Quercus gilva	K. Aoki	02.10.14
35	Kirishimamiike, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.14
		Quercus gilva	K. Aoki	02.10.14
36	Takakuma, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.13
37	Inao, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.13
38	Izashiki, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.13
39	Mt. Taga, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.11
40	Ijuin, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.12
		Castanopsis cuspidata	K. Aoki	02.10.12
41	Higashiichiki, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.12
		Quercus glauca	K. Aoki	02.10.12
42	Kaseda, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.12
43	Noma, Kagoshima, Japan	*Castanopsis sieboldii*	K. Aoki	02.10.12
44	Mt. Omoto, Ishigaki Isl., Okinawa, Japan	*Castanopsis sieboldii* var. *lutchuuensis*	A. Seo	02.11
45	Iriomote Isl., Okinawa, Japan	*Castanopsis sieboldii* var. *lutchuuensis*	A. Seo	02.11

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